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Encasement of *Erysiphe graminis* Haustoria after Treatment with Bromuconazole

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Abstract: Preventive application of bromuconazole caused reduction in size and increased encasement rate of haustoria of *Erysiphe graminis* DC. For example, seven days after inoculation, 60 and 70% of haustoria had been encased in leaves treated with 8 mg litre^{-1} and 16 mg litre^{-1} respectively; the average length of the digitations was 8–10 μm in treated cells compared to 24 μm in untreated cells. The encasement process extended from the neck region to the whole haustorium. Haustorial bodies from treated plants had electron-dense cytoplasm and their organelles were more difficult to identify than in control plants. Extrahaustorial matrix was reduced to an unusually thin, osmiophilic pellicle, surrounded by abundant heterogeneous encasement material. Curative treatment induced similar changes, especially in the margin of the colonies. In the centre of the colony, haustoria were less affected by the fungicide; deposition of collar-like material, modification of extrahaustorial matrix and membrane and accumulation of plant cytoplasm around the digitations resulted in an intermediate, 'swollen' state of digitate haustoria. The possible pathway of encasement events is discussed.

Key words: *Erysiphe graminis*, haustorium, encasement, bromuconazole, fungicide, ergosterol inhibitor, microscopy.

1 INTRODUCTION

Erysiphe graminis DC f. sp. *tritici* Marchal, the causal fungus of wheat powdery mildew, is responsible for important yield losses in wheat worldwide. This pathogen, an obligate parasite, can grow and reproduce only on a living host plant. Extensive information on the biology and histology of the host–parasite interaction[1–11] and on the effects of some chemical compounds[12,13] is available from the literature, but little is known about the reaction of the host–parasite complex to systemic fungicides, particularly at the ultrastructural level.[13]

Two main chemical families of fungicides are effective against powdery mildew fungi: 2-aminopyridines (e.g. ethirimol) and the complex group of the ergosterol biosynthesis inhibitors (EBIs) which include triazole, imidazole and morpholine derivatives.[14–16] In recent years a new triazole-derived fungicide, bromuconazole, 1-((2*RS*,4*RS*;2*RS*,4*SR*)-4-bromo-2-(2,4-dichlorophenyl)-tetrahydrofurfuryl)-1*H*-1,2,4-triazole has been developed which differs from other members of this family in the broadness of its activity spectrum.[17] In preventive treatment at low concentrations, this fungicide, like others in the same group, does not influence the initial stages of the infection but strongly inhibits the formation of haustoria.[18,19]

As previously described for other inhibitors,[13,18,19] bromuconazole induces an encasement phenomenon, a not clearly understood process that results in haustorium inactivation by wrapping in a callose shell. Few cytological investigations, compared to in-vitro studies,[20–24] have analysed fungicide effects on whole host–pathogen interactions. The present study describes the morphological and ultrastructural changes induced by the new triazole fungicide, bromuconazole, with the

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aim of documenting and understanding better the phenomenon of encasement.

2 MATERIALS AND METHODS

Every experiment was performed at least twice.

2.1 Fungal and plant material

Wheat seeds (*Triticum aestivum* L. cv. Talent, a variety with medium sensitivity to powdery mildew disease), were sown in 7 × 7 cm plastic pots containing a locally developed and marketed mixture of equal volumes of peat and ground pozzolana (a porous volcanic rock), as a substrate. Plants were grown in a greenhouse at 18–20°C under 10 000–20 000 lux white light, with a 14-h photoperiod.

Seven-day-old seedlings (first-leaf stage) were inoculated with a strain of *Erysiphe graminis* f. sp. *tritici* by dusting with conidia from plants inoculated 10 days previously. The fungal strain was initially isolated from diseased field-plants from Chazay d'Azergues, France, a place where no resistance to EBI fungicides has been observed, and subsequently maintained in the greenhouse. Inoculated plants were left on the greenhouse bench under 60–70% relative humidity, at 20 (±1)°C; disease symptoms usually appeared on inoculated leaves within seven or eight days in such conditions, but penetration was effective within 12 h and mature haustoria (i.e. digitate) could already be observed after 36 h.

2.2 Fungicide treatment

Application solutions of fungicide at 16, 8 and 4 mg litre^{-1} were made from concentrated stock solutions of technical grade bromuconazole (99.6% purity) in acetone, by diluting with water and adding 'Tween' 80® (polyoxyethylene sorbitan monooleate) to give a final bromuconazole/'Tween' 80 ratio of 2 : 1 by weight. Spraying equipment included a laboratory treatment chamber with turntable and four spraying nozzles. Final concentrations of acetone in the application solutions did not exceed 1 ml litre^{-1}. Control plants were treated with a similar concentration of acetone. Preventive treatment was applied 24 h before inoculation. Curative treatment was applied by spraying three days after inoculation with *E. graminis.*

2.3 Light microscopy

For monitoring the development of *E. graminis.* f. sp. *tritici* after application of the fungicide, the infected primary leaves were harvested four days after the treatment. The whole staining procedure was carried out at room temperature. Leaf segments, about 2 cm long, were excised and cleared in chloroform + methanol (2 + 1 by volume).[25] After 4 h, completely depigmented leaves were transferred to lactophenol + ethanol (1 + 2 by volume) fixative, rinsed three times, (5 min each) in 50% ethanol, then three times in distilled water. Samples were stained first in periodic acid Schiff (PAS) reagent[19] followed by 5 min in lactophenol blue (Merck & Co. commercial preparation) which proved to yield better results than the recommended resorcinol blue. The first stained fungal structures inside the leaves while the second stained surface hyphae, appressoria and conidia.

2.4 Low-temperature scanning electron microscopy

Infected primary leaves were cut from treated and untreated plants, seven days after inoculation with the pathogen (i.e. four days after curative treatment). Observations were made directly on frozen samples at −150°C using the Emscope SP2000 sputter-cryo attachment with a Jeol 35CF scanning electron microscope. The samples were mounted on the support with carbon glue or 'Tissue-tek'® adhesive and then frozen by plunging in liquid nitrogen slush. After 3 min sputter coating with gold-palladium, the samples were transferred into the microscope and examined at 10 kV.

2.5 Transmission electron microscopy

Seven or 13 days after inoculation the inoculated areas of the primary leaves were excised with a razor blade and immediately immersed in buffered paraformaldehyde-glutaraldehyde mixture (glutaraldehyde 20 g litre^{-1}, paraformaldehyde 5 g litre^{-1} 0·2 M McIlvaine citrate-phosphate buffer, pH 6·8) and fixed at room temperature for 4 h. After washing in the same buffer (3 × 1 h), the samples were postfixed in buffered osmium tetroxide at 5 g litre^{-1} overnight. Dehydration was carried out using a graded series of ethanol solutions (250, 500, 700 ml litre^{-1} and finally pure ethanol) followed by epoxy 1–2 propane (three times). Embedding was performed using progressive concentrations of Spurr's resin[26] in propylene oxide (50, 250, 500, 750 ml litre^{-1} for 1 h, then 100% overnight). After transferring to freshly prepared Spurr's mixture, polymerisation was achieved at 60°C for 20 h. Ultrathin sections were cut, using glass knives, on a Reichert-Jung ultramicrotome from three different embedded samples for each treatment and for the two replications of each experiment. Sections were stained with uranyl acetate (70 g litre^{-1} in methanol for 30 min at room temperature) and lead citrate[27] (10 min), and then examined with a Hitachi HU12A microscope operated at 75 kV.

3 RESULTS

3.1 Light microscope observations on haustorium development

3.1.1. Haustorial shape

In control leaves, appressoria forced an infection peg into epidermal cells where it first enlarged to form a globular primary haustorium. Two bundles of digitations then developed at opposite sides of the haustorium (Fig. 1(a)). Three groups of haustoria, globular, digitate and encased, could be distinguished. For each condition 60 haustoria were counted. Table 1 shows the percentage of each type of haustorium found on control and preventively treated plants, three and seven days after inoculation. The haustorial neck and body seemed to be encased first (Fig. 1(b)) since encased haustoria with loose digitations could be observed. Encasement subsequently extended to the digitations (Fig. 1(c)). As Table 1 shows, while the percentages of globular haustoria were similar in treated and untreated plants, the percentage of encased haustoria (both globular- and digitate-encased) was very high in treated plants compared to that in untreated plants. This was particularly evident seven days after inoculation. At the same time the percentage of juvenile haustoria (globular form) or mature haustoria (digitate form) decreased (Table 1).

Curative treatment induced the same kind of modification in haustorial morphology (Fig. 1(d)): seven days after inoculation, the great majority (98%) of the haustorial bodies from untreated plants had differentiated many digitations, compared to only 38% for the treated plants (bromuconazole 8 mg litre^{-1}). In the latter case, the remaining haustorial bodies were completely encased. Digitate haustoria were localised in the centre of the infection while the encapsulated cells were found in the peripheral area. It is likely that these latter haustoria either initiated their development just prior to treatment and were blocked at an immature stage, or attempted to differentiate within fungicide-loaded cells,

TABLE 1
Frequency of Different Haustorial Types of *Erysiphe graminis* Three and Seven Days after Inoculation on Wheat, Untreated and Preventively Treated with Bromuconazole

	Frequency (%)[a]					
	3 days			*7 days*		
	GH[b]	*DH*[b]	*EH*[b]	*GH*	*DH*	*EH*
Untreated	24	75	1	0	98	2
Bromuconazole						
8 mg litre^{-1}	38	39	24	35	5	59
16 mg litre^{-1}	28	61	11	12	17	71

[a] Calculated from 60 observations for each condition.
[b] GH: globular haustoria; DH: digitate haustoria; EH: encased haustoria.

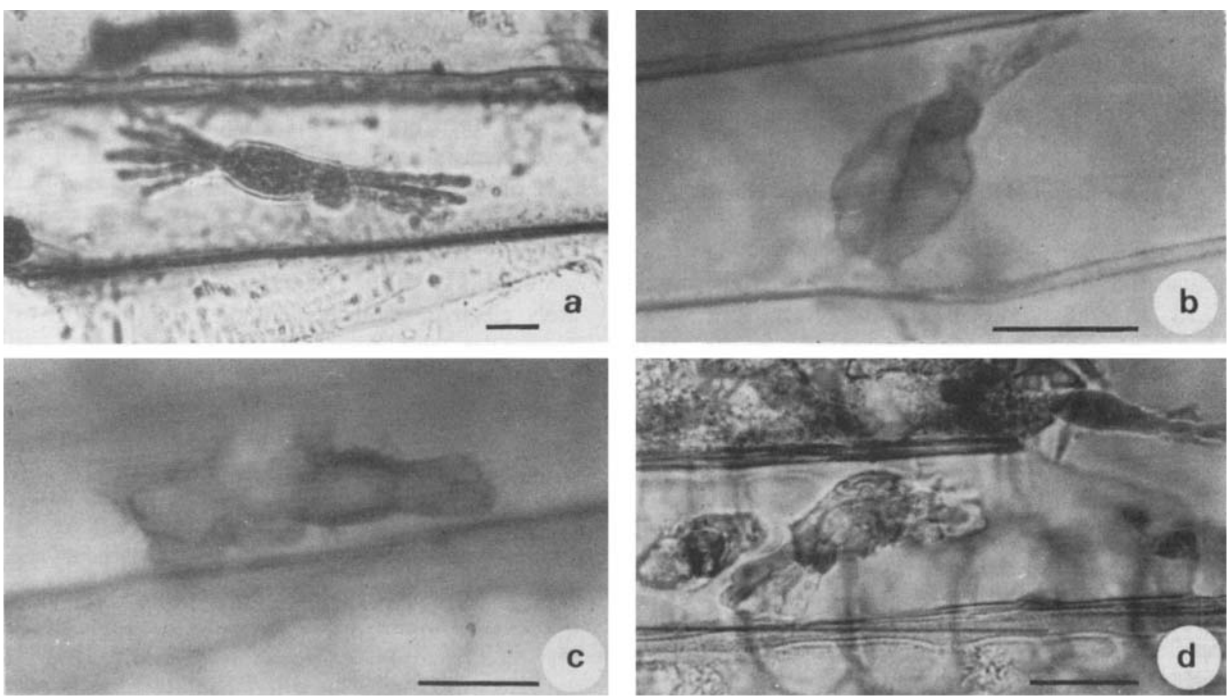


Fig. 1. Light microcopy of untreated and fungicide-treated haustorial cells of *Erysiphe graminis*, stained with PAS and lactophenol blue, bars = 10 μm: (a) untreated control leaf seven days post-inoculation; (b) and (c) leaf preventively treated with bromuconazole 16 mg litre^{-1}: (b) beginning of encasement process, (c) encased digitate haustorium; (d) haustorium in leaf curatively treated (three days post-inoculation) with bromuconazole 16 mg litre^{-1} observed four days later: haustorial body was encased, digitations were not.

TABLE 2

Mean Sizes of Haustorial Bodies and Digitations of *Erysiphe graminis* Three and Seven Days after Inoculation on Wheat Leaves, Untreated and Preventively Treated with Bromuconazole

	Mean size (μm) (±5% CI)[a]			
	3 days		*7 days*	
	HB[b]	*HD*[b]	*HB*	*HD*
Untreated	14·7 (±1·28)	20·0 (±1·97)	17·6 (±0·6)	24·0 (±1·82)
Bromuconazole				
8 mg litre^{-1}	11·4 (±1·28)	9·1 (±1·98)	12·8 (±1·90)	8·0 (±1·40)
16 mg litre^{-1}	12·6 (±1·76)	8·1 (±2·22)	14·8 (±1·90)	7·8 (±1·32)

[a] calculated from 50–60 individual measurements.
[b] HB: haustorial bodies; HD: haustorial digitations.

after treatment, i.e. under conditions of preventive fungicide application.

3.1.2 *Haustorial size*

Modifications induced by preventive treatment were quantified by measurements of the size of haustorial bodies and digitations (Table 2). Three and seven days after inoculation, non-encased haustorial bodies from treated plants were smaller, and their digitations much shorter, than those in control tissue. Similar observations were made after curative application (not shown).

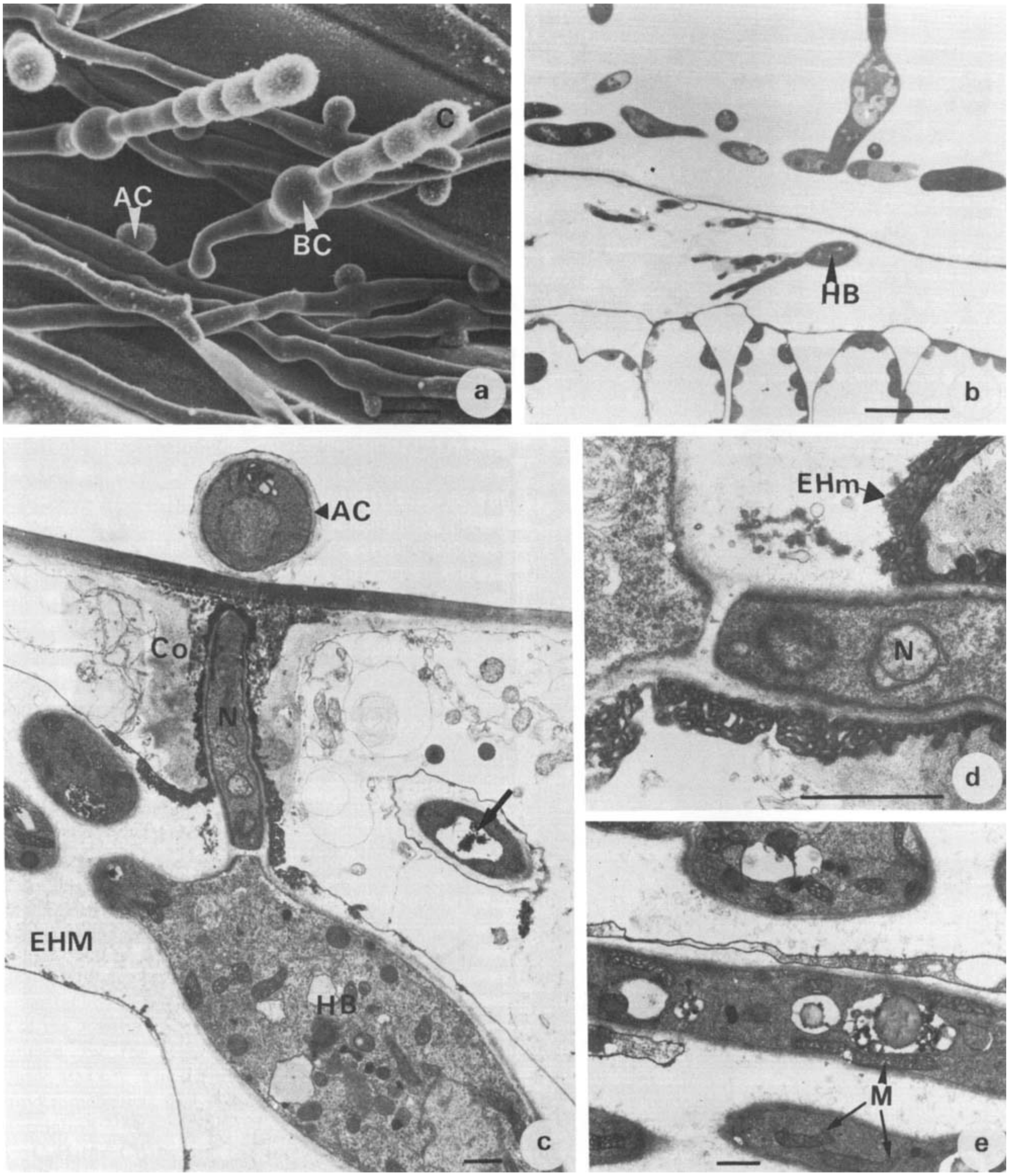
3.2 Ultrastructural observations

Haustoria in the control (i.e. untreated) wheat leaves had generally digit-shaped appendages when mature (Fig. 2(b)). Central bodies with single nuclei and numerous digitations containing osmiophilic bodies (Fig. 2(c), 2(e)) were observed. Fungal walls around haustoria had a fibrillar structure, fibrils being looser and perpendicular to the wall-plane on the outer side (Fig. 2(d)). The extrahaustorial matrix, the volume of which could vary, contained generally amorphous materials, and was limited by a membrane with increased opacity, the extrahaustorial membrane (Fig. 2(c)). This membrane was highly convoluted and folded, particularly in the neck region (Fig. 2(d)). Observations have been made[18] on treated non-infected wheat leaves to check for possible modifications in the cell structure that the fungicide could induce. No ultrastructural differences appeared between untreated plants and plants treated with bromuconazole at 32 mg litre^{-1} (not illustrated).

After treatment with bromuconazole at 4 mg litre^{-1} (Figs 3(a)–3(e)) the infection pegs derived from highly vacuolated superficial appressoria, which resulted from swelling of the apices of superficial hyphae (Fig. 3(b)). The hyphal walls of these appressoria had frequently an irregular profile. Also, in the centre of the infection, where the treatment induced less damage to haustorial cells (Fig. 3(a)), the extrahaustorial matrix material surrounding digitations contained a fibrillar-granular, slightly opaque substance (Fig. 3(c)). Within the digitations, stroma of mitochondria appeared granular and swollen as compared to the control (Figs 3(c), 3(e)). Cristae were less numerous and poorly defined. Host cytoplasm contrast was increased. At a higher magnification, it exhibited numerous vesicular and multivesicular bodies, associated with dilated smooth reticulum. At this level, the treatment seemed to induce an increase in biosynthesis, resulting in deposition of collar-like material together with modifications of the extrahaustorial matrix (Fig. 3(e)), and accumulation of plant cytoplasm around the digitations which increased their thickness and finally gave the swollen aspect of digitate haustoria observed by light microscopy.

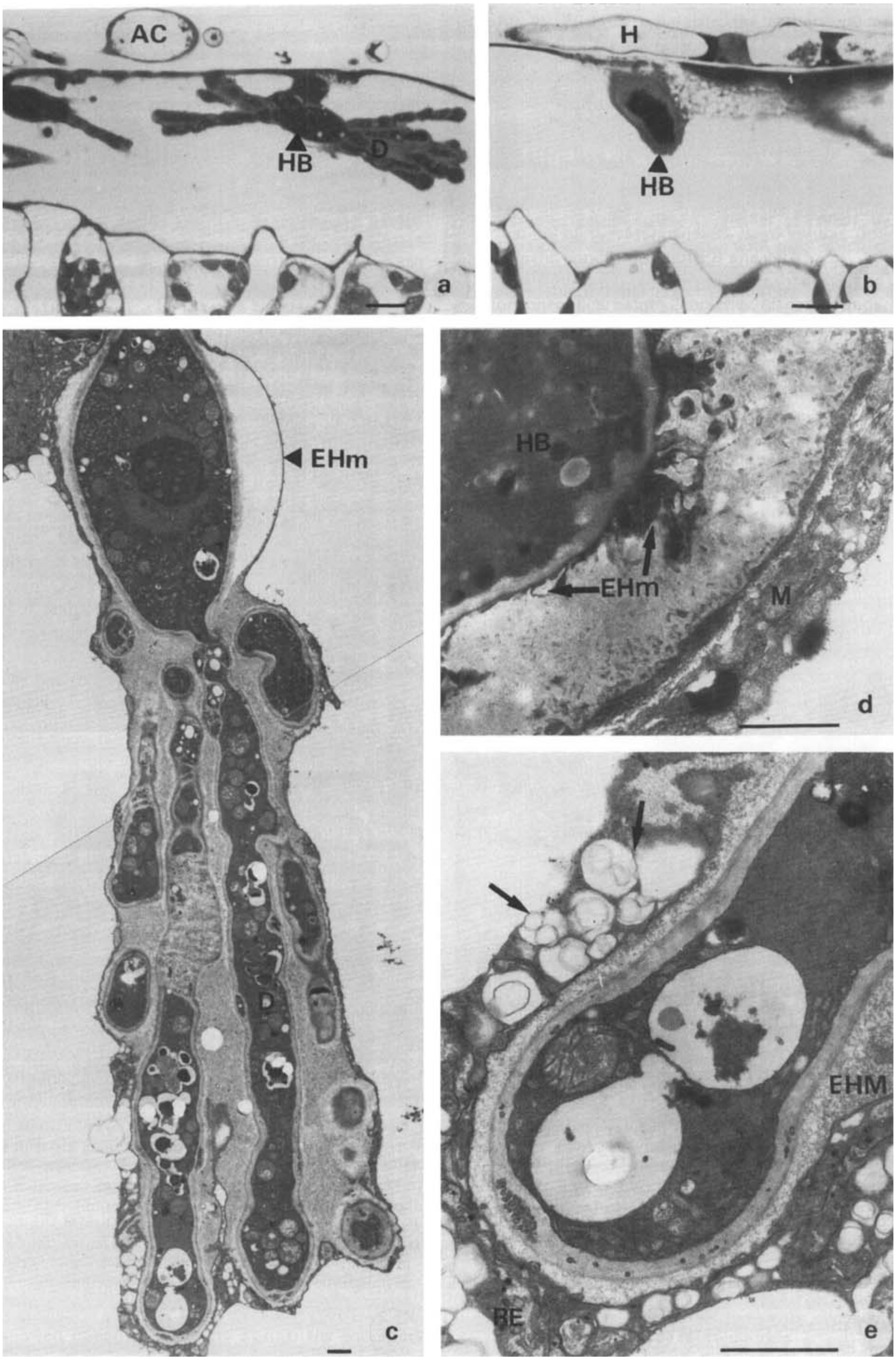
In the peripheral area, haustoria were greatly modified by the fungicide treatment (Figs 3(b), (3d)). The cytoplasm of haustorial bodies appeared electron-dense and organelles within were difficult to identify. Extrahaustorial matrix was restricted to an osmiophilic thin pellicle, surrounded by heterogeneous encasement material (Fig. 3(d)). The latter was composed of moderately electron-dense cement containing darkly stained corpuscles and fragments that appeared similar to extrahaustorial membrane, from which they may have derived. A multi-layered collar was installed around the haustorial neck (Fig. 3(c)). The epidermal cell wall, particularly the outermost layer, was densely stained. Superficial hyphae were necrotic, with fragmented cytoplasm and no visible organelles.

Following treatment with bromuconazole at 8 mg litre^{-1} (Figs 4(a)–4(c)), modifications were similar to those at 4 mg litre^{-1}, but with increased intensity. Haustoria in the peripheral area of the colonies were encased (Figs 4(a), 4(b)). Haustorial bodies contained compact cytoplasm, where endoplasmic reticulum and ribosomes were difficult to identify. The walls of haustorial bodies exhibited increased thickness. The extrahaustorial matrix was thin and electron-dense;



Key to abbreviations on figures: AC, appressorial cell; BC, basal cell, C, conidia; Co, collar; D, digitation; EHm, extrahaustorial membrane; EHM, extrahaustorial matrix; H, hyphae; HB, haustorial body; LTSEM, low temperature scanning electron microscopy; N, Neck of haustorium; M, mitochondrion; RE, reticulum; TEM, transmission electron microscopy. Staining solutions: Fig. 2(b) and 3(a, b): methylene blue/azure II; Fig. (2c, d, e) 3(c, d, e) and Fig. 4(b): uranyl acetate and lead citrate.

Fig. 2. Light and electron microscope views of untreated leaves seven days infected by *Erysiphe graminis*. (a) and (b), bars = 20 μm: (a) infection viewed by LTSEM; (b) longitudinal section observed in light microscopy, note the thickness of the digitations of the haustorial cell; (c), (d) and (e) TEM views, bars = 1 μm: (c) longitudinal section through appressorium, collar, haustorial neck and haustorial body; haustorial digitations with osmiophilic bodies (→); (d) high magnification of haustorial neck; note folding and convolutions of extrahaustorial membrane; (e) detail of haustorial digitation, mitochondria with enlarged profile.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extensions, or remnants, most likely related to the matrix layer were visible within the surrounding encasement material (Fig. 4(c)). The latter appeared to be made of a fibrillar network, with the interfibrillar space obstructed by electron-dense substance. Occurrence of mitochondria and rough reticulum indicated active contribution of the epidermal cell to the encasement of haustoria (Fig. 4(b)). All these modifications resulted in the absence of conidial formation on superficial hyphae (Fig. 4(a)) unlike in the control (Fig. 2(a)).

4 DISCUSSION

Although we have previously observed the encasement phenomenon of ageing haustoria in a variety of species, particularly powdery mildew fungi,[18] only 2% encased haustoria were observed in untreated leaves seven days after inoculation (Table 2). This study of the effect of bromuconazole on the wheat powdery mildew host–pathogen interaction showed the most conspicuous reaction to be the earlier encasement of haustoria under any treatment conditions.

In electron microscope observations, the occurrence of abundant endoplasmic reticulum suggested enhancement of metabolic processes that could be involved in the encasement process. Following the lower dosage of fungicide (4 mg litre^{-1}), the presence of a cytoplasmic area with a high content of endoplasmic reticulum and vesicular bodies, near to the digitations, which showed signs of damage (swollen mitochondria, storage vacuoles), indicated a high level of biosynthesis involved in the encasement of the digitations. In *Leveillula taurica* (Lev.) Arn., after acridine orange staining, it was shown that the material which encases the haustorium was in continuity with the papilla.[28] Encasement material was said to derive from collar extension in *Erysiphe pisi* DC;[29] similar results were observed for an incompatible host–pathogen interaction.[30]

Our observations with light microscopy and examination of thin sections led us to propose that the elaboration of encasement depended on active secretion from the host cell, resulting in collar extension which finally encased the whole haustorium. However, complete encasement was fully achieved by synthesis of similar material around digitations in mature haustoria. At the end of the development, the collar-like material enveloped whole haustorial cells and this layer was separated from the protoplasm of the host epidermal cell by the plant plasmalemma.

It can be assumed that when the encapsulation process began, the extrahaustorial membrane was pushed out against the haustorial wall and compressed the extrahaustorial matrix into a thin electron-dense layer. Examination of encapsulation by electron microscopy showed that it was made of two layers of different texture. A similar structure has been observed around haustorial bodies of *Peronospora tabacina* Adam developing into both metalaxyl-treated or untreated plants;[31] although the amount of double-layer encased haustoria was greater than in untreated leaves, the material in both had a positive reaction for callose and cellulose staining. After preventive treatment with bromuconazole, globular or digitate-encased haustoria stained similarly with PAS and lactophenol blue in treated or untreated leaves. We assume, as did other authors,[1,32,33] that these represented ageing haustoria being encapsulated by epidermal cells. The fungicide could thus induce an acceleration of the ageing process. Moreover, since damage in the extrahaustorial membrane was not observed in the normal infection process, the fungicide could also have interfered with plant metabolism. Numerous reports[19,31,34] describe similar observations of haustorial encasement in biotrophic parasites, after treatment with fungicides. According to some authors,[31,34] several non-triazole fungicides are able to induce encasement of haustoria. Deposition of electron-dense material around haustoria in the *Phaseolus vulgaris–Uromyces phaseoli* host–parasite interaction after treatment with oxycarboxin has been observed.[35] In that work it was assumed that encasement was induced by the formation of a non-functional primary haustorium and the haustorium was considered to act as a storage place for metabolites, which could adsorb or accumulate nutrients from the plant as well as toxic substances. In our study, curative application of fungicide induced the deposition of material around differentiating young haustoria. The fungicide seemed to have acted mainly on young or developing haustorial cells. On the other hand, its action was much more limited on differentiated structures. This could explain why young haustoria are more readily encased than the digitate form. The mature haustoria showed only ultrastructural modifications and no encasement phenomenon. However, some haustoria in the intermediate stage could still express the effect of the absorption of bromu-

Fig. 3. Light and electron microscope views of seven-day-old infections on leaves by *Erysiphe graminis* curatively treated (three days after inoculation) with bromuconazole at 4 mg litre^{-1}. (a) and (b) light microscope views of longitudinal section, bars = 10 μm: (a) in haustorium from the centre of infection, (b) in abortive haustorium from the peripheral area, no digitation visible. (c), (d) and (e) TEM views, bars = 1 μm: (c) longitudinal section through digitate haustorium; compare to Fig. 3(a); (d) encased abortive haustorium, as seen in 3(b), collar-like material surrounding haustorial body, extrahaustorial membrane applied against haustorial body wall, with extensions into encasement material; (3) detail of Fig. 3(c), distal part of a digitation, showing increased electron density and volume of extrahaustorial matrix, abundant reticulum profiles and vesicles in cytoplasm of the host cell, and increased physiological activity in the extrahaustorial membrane.

conazole, thus leading to encased haustoria with short digitations. It has been suggested that aged haustoria had lost the capacity to accumulate sufficient fungicide to allow the encasement process.[36] Moreover, after curative treatment, the haustorial cell is exposed to a double attack by the fungicide: directly by absorption of the fungicide from the plant cell as well as from the connected extracellular hyphae.

During development, the pathogen behaves in a similar way in triazole-treated plants and in untreated, resistant wheat cultivars. According to Heath,[37] the encasement process could be a response to toxic substances in which the host recognises the haustorium as a foreign body. Accordingly, the host's resistance to the infection process appears to increase in response to fungicide treatment and, thus, the effect of fungicide application can be considered as the expression of an induced resistance.[36]

Papillae at infection peg formation sites can be interpreted as an important defence mechanism. In additional experiments (data not shown), preventive treatment by bromuconazole did not affect the number of papillae formed. This means that the penetration by the fungus was not inhibited. Similar results were obtained after treatment by triadimefon or triadimenol.[36] Even though the number of papillae formed did not increase after foliar application of bromuconazole, an enhanced synthesis of papillar components was induced after penetration by the pathogen, since larger and thicker papillae were observed. This result suggests that the fungicide action began when the fungus penetrated into the epidermal cell.

In conclusion, this study has demonstrated that haustorial encasement by material partially derived from collar extension was responsible for inhibition of the parasite's growth. After application of a sterol biosynthesis inhibitor fungicide, the deposition of this double-layered wall was strongly enhanced or accelerated.

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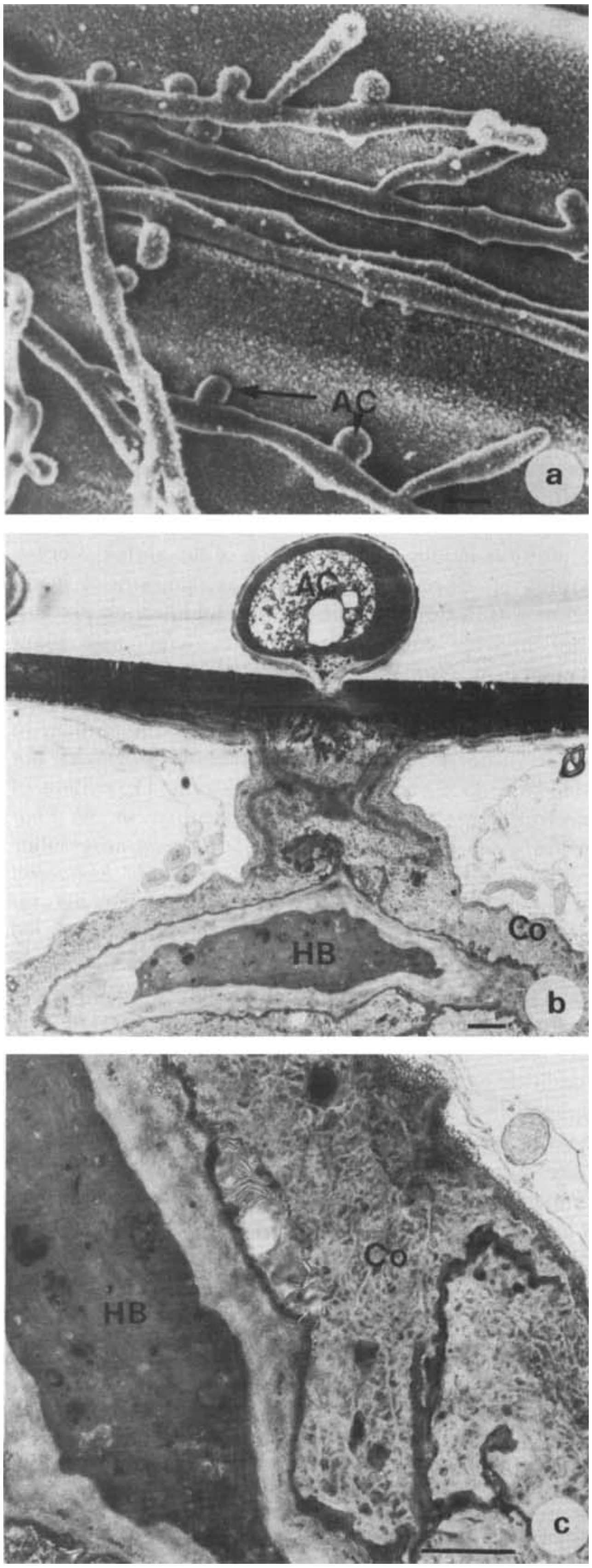

Fig. 4. Electron microscope views of seven-day-old *Erysiphe graminis* infection, curatively treated (three days after inoculation) by bromuconazole at 8 mg litre^{-1}. (a) LTSEM, bars = 10 μm, appressorium possibly differentiated after treatment with bromuconazole; note the absence of conidiophores and conidia; (b) and (c) TEM views, bars = 1 μm: (b) tangential section through vacuolated appressorium and haustorial cell affected by the treatment; collar extension encasing haustorium, with electron-dense material associated with folding of extrahaustorial membrane; (c) detail of encasement material.

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